



## CHANGES

### IN THE SPECIFICATION:

**[0002]** The invention relates to a vWF protease-containing composition which includes a polypeptide comprising the amino acid sequence AAGGILHLELLV (SEQ ID NO: 1), as well as to nucleotide sequences coding for such a polypeptide. It further relates to methods for increasing the stability of the vWF protease.

**[0011]** We have isolated a composition exhibiting vWF protease activity that is capable of proteolytically processing vWF in a physiological manner. Said composition comprises at least one single peptide chain having a molecular weight between 190 kD and 100 kD as determined by SDS PAGE and comprises the sequence AAGGILHLELLV (SEQ ID NO: 1). This amino acid sequence is located at the N-terminus of the peptide chain. The composition comprising the sequence AAGGHILHLELLV (SEQ ID NO: 1) can also be used for isolation, detection or purification of proteins, i.e. von Willebrand Factor.

**[0012]** Furthermore, an isolated polypeptide having a molecular weight between 190 kD and 100 kD according to SDS-PAGE and comprising the sequence AAGGILHLELLV (SEQ ID NO: 1) is also provided. This sequence is preferably directly followed by the sequence AVG, which is preferably followed by the sequence PDVFQAHQEDTERYVLTNLNIGAELLRDP SLGAQFRVHLVK MVILTEPEGAPNITANLTSSLLSVCGWSQTINPEDDTPGHADLVLYITRFDLELPDGNRQVRG VTQLGGAC SPTW SCLITEDTGFDLGVTI (SEQ ID NO: 2).

**[0013]** Another aspect of the present invention is a method of purifying von Willebrand factor comprising contacting a solution containing von Willebrand factor with a substrate comprising the amino acid sequence AAGGILHLELLV (SEQ ID NO: 1) under conditions sufficient to bind von Willebrand factor to the substrate.

Please replace paragraphs 0015 and 0016 with the following:

**[0015]** In addition, the present invention includes a method of processing recombinantly produced vWF through the use of the vWF protease of the present invention, in order to produce a vWF product.

## BRIEF DESCRIPTION OF THE DRAWINGS

Fig.1 shows the schematic purification scheme of the vWF protease containing composition of the present invention.

Fig. 2 shows the partial nucleotide (SEQ ID NO: 3) and amino acid (SEQ ID NO: 4) sequence of the vWF protease of the present invention.

[0016] A composition is provided containing a vWF protease consisting of a polypeptide chain with an apparent molecular weight in SDS-PAGE of around 180 kD, around 170 kD, around 160 kD, around 120 kD, around 110 kD or mixtures of these chains, said chain comprising an amino acid sequence AAGGILHLELLV (SEQ ID NO: 1). Alternatively, this amino acid sequence can be directly followed by the amino acid sequence AVG. Furthermore, this sequence is followed by the sequence PDVFQAHQEDTERYVLTNLTNIGAELLRDPSLGAQFRVHL VKMVILTEPEGAPNITANLTSSLLSVCGWSQTINPEDDTPGHADLVLYITRFDLELPDGNRQV RGVTLGGACSPWWSCLITEDTGFDLGVTI (SEQ ID NO: 2).

Please replace paragraph 0020 with the following:

[0020] The AAGGILHLELLV (SEQ ID NO: 1) sequence is located at the N-terminal region of the protein. Shortening of the peptide chain occurs at the C-terminus or via endoproteolytical cleavages. The composition according to the present invention contains a vWF cleaving protease that is expressed as a single chain protein.

[0022] The peptides containing the amino acid sequence AAGGILHLELLV (SEQ ID NO: 1) or AAGGILHLELLVAVG (SEQ ID NO: 5) or AAGGILHLELLVAVGPDVFQAHQEDTERYVLTNLTNIGAELLRDPSLGAQFRVHLVKMVILTEPEGAPNITANLTSSLLSVCGWSQTINPEDDTPGHADLVLYITRFDLELPDGNRQVRGVTLGGACSPWWSCLITEDTGFDLGVTI (SEQ ID NO: 4) or RRAAGGILHLELLVAVGPDVFQAHQEDTERYVLTNLTNIGAELLRDPSLGAQFRVHLVKMVILTEPEGAPNITANLTSSLLSVCGWSQTINPEDDTPGHADLVLYITRFDLELPDGNRQVRGVTLGGACSPWWSCLITEDTGFDLGVTI (SEQ ID NO: 6) can also be used as tool for detecting proteins binding to the vWF protease or target sites for ligand development for detecting, isolating and purifying proteins that bind to the vWF protease. Preferably the protein to be detected or purified is vWF.

**[0024]** Furthermore, the peptide having the amino acid sequence AAGGILHLELLV (SEQ ID NO: 1) or AAGGILHLELLVAVG (SEQ ID NO: 5) or AAGGILHLELLV

AVGPDVFQAHQEDTERYVLTNLNIGAELLRDPSLGAQFRVHLVKMVILTEPEGAPNITA  
NLTSSLLSVCGWSQTINPEDDDTPGHADLVLYITRFDLELPDGNRQVRGVTQLGGACS  
PTWSCLTEDTGFDLGVTI (SEQ ID NO: 4) or RRAAGGILHLELLV  
AVGPDVFQAHQEDTERYVLTNLNIGAELLRDPSLGAQFRVHLVKMVILTEPEGAPNITA  
NLTSSLLSVCGWSQTINPEDDDTPGHADLVLYITRFDLELPDGNRQVRGVTQLGGACS  
PTWSCLTEDTGFDLGVTI (SEQ ID NO: 6) can also be used for the development of anti-vWF protease antibodies using techniques as known from the art. The development of these antibodies or antibody derivatives or peptidomimetics can be accomplished according methods known to the prior art (Greer J. et al., J.Med.Chem., 1994, Vol. 37, pp. 1035-1054; Harlow E. and Lane D., in "Antibodies. A Laboratory manual", Cold Spring Harbor Laboratory, 1988, Esser C. and Radbruchj A., Annu. Rev. Immunol., 1990, vol. 8, pp. 717-735; Kemp D.S., 1990, Trends Biotechnol., pp. 249-255).

**[0025]** The present invention relates also to single polypeptide chains having an apparent molecular weight in reduced SDS-PAGE of between 190 kD and 100 kD, preferably about 180 kD, more preferably about 170 kD, in a particularly preferred embodiment about 160 kD, preferably 120 kD and most preferably about 110 kD, comprising an N-terminal amino acid sequence AAGGILHLELLV (SEQ ID NO: 1). This sequence is preferably directly followed by the sequence AVG, which is then preferably followed by the sequence PDVFQAHQEDTERY VLTNLNIGAELLRDPSLGAQFRVHLVKMVILTEPEGAPNITANLTSSLLSVCGWSQTINPEDDDTPGHADLVLYITRFDLELPDGNRQVRGVTQLGGACSPTWSCLTEDTGFDLGVTI (SEQ ID NO: 2). In contrast to the protease entities described by Furlan et al. (1996) and Tsai (Blood 87(10) (1996), pp.4235-4244) the vWF multimerase entities according to the present invention are much smaller than the entities described in these documents (around 300 kD (Furlan et al.), and 200 kD (Tsai), respectively).

**[0052]** The peptide with the sequence AAGGILHLELLV (SEQ ID NO: 1) was synthesized on a solid-phase support following the method of Barany, G and Merrifield, R.B. (1980) Solid-phase Peptide Synthesis, in *The Peptides* vol. 2 (Gross, E. and Meienhofer, J., eds) Academic, New York. After cleavage and de-protection of the peptide, the peptide was purified by ion-exchange chromatography. The peptide was characterized by reverse phase HPLC on a C8 silica column with gradient elution in trifluoro acetic acid with acetonitrile. The peptide showed no major byproducts.

**[0056]** The peptide with the sequence AAGGILHLELLV (SEQ ID NO: 1) was synthesized and purified as described in example 2. The peptide was then used to immunize 3 months old BALB/c mice with the following protocol: A primary subcutaneous injection of 100 µg peptide antigen emulsified in Freund's complete adjuvant in 100 µl followed by intra-peritoneal boosts of 100 µg peptide antigen in phosphate buffered saline at monthly intervals.

**[0057]** The anti-peptide titer was tested by routine ELISA method using purified peptide as screening antigen. After the final boost the spleens were taken from the mice for cell fusion. Cell fusion was carried out according to a standard protocol originally described by Kohler G. and Milstein C. 1975, *Nature* 256:495. Anti-peptide antibodies producing hybridoma cell lines were screened by standard techniques with the purified peptide as screening antigen essentially based on a conventional ELISA methodology. After cloning a cell line could be isolated with a high expression level of an antibody specific for the screening peptide with the sequence AAGGILHLELLV (SEQ ID NO: 1). This cell line was cultured on serum-free culture medium and grown to high density. The supernatant of the cell culture was harvested by centrifugation to remove cells and the monoclonal antibody containing supernatant was concentrated by ultra-diafiltration and conditioned for further use.

**[0058]** The monoclonal antibody obtained had a high selectivity for the vWF cleaving protease as described by Furlan et al. 1996, *Blood* 87:4223-4234. This monoclonal antibody was immobilized to a polystyrene ELISA plate in a carbonate/bi-carbonate buffer, 0.05 molar, pH 9.6, at a concentration of 5 µg immunoglobuline/ml overnight (16 hours) at 4°C, with each 100 µl of coating solution per well. The coating solution was removed from the wells and replaced by a solution of bovine serum albumin (BSA) at a concentration of 100 µg/ml at a volume of 100 µL per well, for 2 hours. The BSA solution was removed and the wells were washed with phosphate buffered saline. The pre-coated plates were then incubated with either

samples of platelet poor plasma from healthy human plasma donors or platelet poor plasma from patients with an unclear diagnosis of either thrombotic thrombocytopenic purpura (TTP) or hemolytic uremic syndrome (HUS). After incubation of the plasma samples with the antibody coated ELISA plates as in a routine sandwich ELISA system, after 3 hours the plasma was removed from the wells. Wells were washed with phosphate buffered saline and incubated with the monoclonal antibody directed against the peptide with the sequence AAGGILHLELLV (SEQ ID NO: 1), conjugated with horse radish peroxidase following the method of Wilson, M.B. and Nakane, P.K. (1978) In *Immunofluorescence and Related Staining Techniques*. Knapp, W. Holubar, K. and Wick, G (eds), Elsevier/North Holland, Amsterdam, p. 215, and detected by the OPT reagent as described by Cathy D. and Raykundalia Ch. (1989) ELISA and related enzyme immunoassays, in *Antibodies II a practical approach*. Cathy D (ed), IRL Press Eynsham Oxford England, p. 97.

Please replace Table 1 with the following:

Molecular weight	Amino Acid sequence
350 kDa unreduced	Ser-Val-Ser-Gly-Lys-Pro-Gln-Tyr-Met-Val* ( <u>SEQ ID NO: 7</u> )
150 kDa unred.	Ala-Ala-Gly-Gly-Ile ( <u>SEQ ID NO: 8</u> )
140 kDa unred.	Ala-Ala-Gly-Gly-Ile ( <u>SEQ ID NO: 8</u> )
130 kDa unred.	Ala-Ala-Gly-Gly-Ile ( <u>SEQ ID NO: 8</u> )
110 kDa unred.	Ala-Ala-Gly-Gly-Ile-Leu-His-Leu-Glu ( <u>SEQ ID NO: 9</u> )
70 kDa unred.	Asp/Ser-Gln/Leu-Thr/Met-Val/Pro-Ser/Phe** ( <u>SEQ ID NO: 10</u> )
180 kDa reduced	Ala-Ala-Gly-Gly-Ile-Leu-His-Leu-Glu ( <u>SEQ ID NO: 9</u> )
170 kDa reduced	Ala-Ala-Gly-Gly-Ile ( <u>SEQ ID NO: 8</u> )
160 kDa reduced	Ala-Ala-Gly-Gly-Ile-Leu-His-Leu-Glu-Leu-Leu-Val-Ala-Val-Gly ( <u>SEQ ID NO: 11</u> )
120 kDa reduced	Ala-Ala-Gly-Gly-Ile-Leu-His-Leu-Glu-Leu-Leu-Val-Ala-Val-Gly ( <u>SEQ ID NO: 11</u> )
40 kDa reduced	Asp-Gln-Thr-Val-Ser** ( <u>SEQ ID NO: 12</u> )